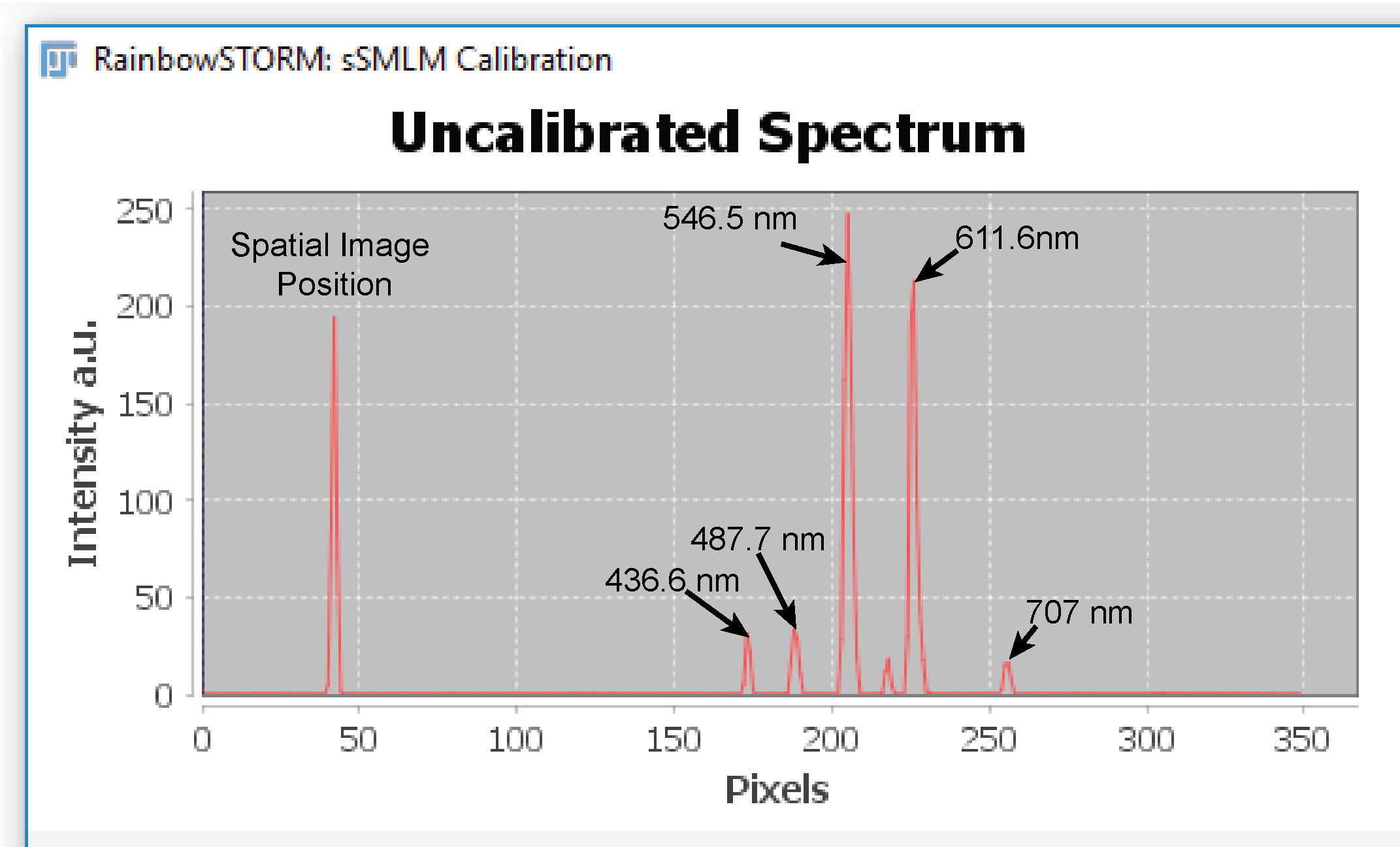
RainbowSTORM Test Data

## Install RainbowSTORM

## Download the RainbowSTORM plugin (rs\_ij\_plugin-0.1.0-SNAPSHOT.jar)

* 1. Copy the file into the Plugins subfolder of your ImageJ installation (e.g. “C:\Program Files\ImageJ\plugins”)
  2. Verify the successful installation of RainbowSTORM
  3. Restart ImageJ
  4. Locate RainbowSTORM under the Plugins menu
  5. Download and install the latest version of ThunderSTORM

1. Access Help Screens
   1. Launch the RainbowSTORM Help Screen (from the ImageJ menu Plugins🡪RainbowSTORM🡪RainbowSTORM Help)
   2. Use hyperlinks to view help for each RainbowSTORM screen
   3. Help pages for each screen can be accessed by pressing the blue question mark icon on each screen.
2. Calibrate the System
   1. Download the calibration images (Calibration.tif)
      * Calibration Image Properties;
        + Calibration Source: Calibration Lamp
        + Dispersive Element: Grating
   2. Open ImageJ
   3. Drag the calibration images into the ImageJ window or load the calibration images
   4. Launch the RainbowSTORM Calibration Screen (from the ImageJ menu Plugins🡪RainbowSTORM🡪sSMLM Calibration)
   5. Double-click on the line plot displayed in the uncalibrated spectrum graph to update the value of the current peak value text field starting with the first peak representing the spatial peak position). Click and drag to zoom in on an area.
   6. Press the 'Set' button to update the activated text field. Use the 'Previous' and 'Next' buttons to change which text field is activated.
   7. Select the spectral peaks for example the default wavelengths correspond to the spectral peaks at 487.7 nm, 546.5 nm and 611.6 nm indicated below:



**Figure 1:** Example of spatial and spectral peaks for the example calibration image

* 1. The default settings for the calibration source (calibration light source) and the dispersive element(grating) are appropriate for these calibration images
  2. Press the 'Calibrate' button
  3. Save the resulting pixel positions, corresponding wavelength information, and fitting method
  4. Close the RainbowSTORM Calibration screen

1. Analyze the sSMLM Images
   1. Download the sSMLM images (xx.tif)
   2. Drag the sSMLM images into the ImageJ window or load the sSMLM images
   3. Launch the RainbowSTORM sSMLM Analysis Screen (Plugins🡪 RainbowSTORM🡪sSMLM Analysis)
   4. Set the camera parameters
      * Camera pixel size: 160 nm
      * adu: 4.6
      * Offset: 200
      * EM Gain: 100
   5. Use the Rectangular Selection tool from ImageJ's toolbar to select the spatial image in the sSMLM image or manually input the parameters of the rectangle into the text fields (initial x position, initial y position, rectangle width, and rectangle height) on the sSMLM Analysis screen
   6. Press the 'Crop sSMLM Image' button on the sSMLM Analysis screen to separate the sSMLM images into spatial and spectral images
   7. Optional-Press the 'Save Parameters' button to save the camera setup and cropping parameters (use an appropriate name to prevent overwriting other RainbowSTORM files)
   8. Select the Spatial Region (Cropped Region 1) and open ThunderSTORM
   9. Make sure the camera settings used in ThunderSTORM and RainbowSTORM are the same
   10. Analyze the spatial images using ThunderSTORM (the default settings are appropriate)
   11. Save the results (don't overwrite any of the previously saved files)
       * Optional- deselect unwanted fields (Required ThunderSTORM fields: frame, x, y, intensity, sigma and uncertainty)
   12. Close ThunderSTORM
   13. Load ThunderSTORM results into RainbowSTORM
   14. Load Calibration Information into RainbowSTORM
   15. With the Automatic Background Subtraction' checkbox, press the 'Subtract' button
   16. Default settings are optimized for Far Red dyes e.g. Alexa Fluor 647
   17. Optional - Check the 'Remove Overlapping Spectra' checkbox to excluded localizations with overlapping spectra
   18. Optional - Preview the results of the spectroscopic analysis for individual localizations
   19. Press the 'Run Analysis' to calculate the results of the spectroscopic analysis for all localizations
   20. Select a field (e.g. spectral sigma) from the histograms of the localizations for each RainbowSTORM field can be generated by selecting the field and pressing the "Show Histogram" button.
   21. Use the selected histogram to identify and a minimum and maximum value for the Filter Range. Input the values(e.g. spectral sigma 16 nm to 23 nm) and press the ‘Apply Filter’ button.
   22. Press the ‘Reset sSMLM Data’ button to remove filter results
   23. Update the Visualization settings:
       * Reconstruction method: Averaged Gaussian
       * Magnification:1
   24. Press the ‘Calculate FRC’ to show results from Fourier Ring Correlation Analysis
   25. Close FRC Results
   26. Update the Visualization settings:
       * Reconstruction method: Averaged Gaussian
       * Magnification: 5
   27. Use the ImageJ's rectangular selection tool is used to select a region of interest (ROI) in the current sSMLM reconstruction then pressing the "Restrict to ROI" button to select localizations within the ROI.
   28. Press the "Classify by Centroid" button launches the RainbowSTORM Classification screen where localizations can be grouped into color-coded image windows based on their spectral centroids.
       * Set three spectral windows (W1: 680-689, W2:692-698 and W3:703-709)
       * Optional - Check the ‘Show Channel’ checkbox to display a reconstruction of each individual channel
   29. Press the ‘Save Spectroscopic Data’ button to save the current spectroscopic data.
   30. Close the Visualization screen.
2. Import previously saved data
   1. Launch the Import Screen
      * Select Plugins🡪 RainbowSTORM🡪sSMLM Analysis from the ImageJ menu
   2. Load the Data: Previously saved data files can be loaded into RainbowSTORM by selecting the file ending in "\_spec.csv".
   3. Set Camera Setup and Cropping Parameters: The camera setup parameters and the cropping can be manually input, or the previously saved parameters can be loaded.
   4. Press the ‘Visualize Data’ button to render the Lambda-colored image and load the data into the Visualization Screen.
3. Launch Help Screens